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# Simple fibre based dispersion management for two-photon excited fluorescence imaging through an endoscope

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## ABSTRACT

We want to implement two-photon excitation fluorescence microscopy (TPEFM) into endoscopes, since TPEFM can provide relevant biomarkers for cancer staging and grading in hollow organs, endoscopically accessible through natural orifices. However, many obstacles must be overcome, among others the delivery of short laser pulses to the distal end of the endoscope. To this avail, we present imaging results using an all-fibre dispersion management scheme in a TPEFM setup. The scheme has been conceived by Jespersen et al. in 2010<sup>1</sup> and relies on the combination of a single mode fibre with normal and a higher order mode fibre with anomalous dispersion properties, fused in series using a long period grating. We show that using this fibre assembly, a simple and robust pulsed laser delivery system without any free-space optics, which is thus suitable for clinical use, can be realised.

**Keywords:** two-photon microscopy, femtosecond laser pulse, dispersion management

## 1. INTRODUCTION

Two-photon excitation fluorescence microscopy (TPEFM) is a powerful technique for visualizing tissue on a cellular and sub-cellular scale. Moreover, TPEFM offers a number of advantages compared to other techniques such as confocal and wide-field microscopy. First and foremost, the near infrared light used to excite the two-photon fluorescence experiences less scattering in tissue than green or blue light, typically used for single photon fluorescence excitation or white-light imaging, resulting in a larger imaging depth with TPEFM.<sup>2,3</sup> There is also an abundance of endogenous fluorescent substances that can be two-photon excited, such as NADH and FAD, and they can thus readily be used as biomarkers with diagnostic value. Also, in TPEFM, fluorophore excitation occurs only at a small volume around the focal point of the objective, providing intrinsic 3D resolution and reducing the risk for photodamage to the surrounding tissue. Additionally, there is a large Stokes shift between emission and excitation spectrum, which enables simple filtering of the fluorescence signal from the excitation light. All these properties make TPEFM an attractive option for clinical use, where it could provide previously inaccessible biomarkers to improve or even enable early diagnosis.

However, since two-photon excitation cross sections of fluorophores are typically very small, a high irradiance is needed to generate a fluorescence signal. The preferred method is to use a mode-locked laser, such as a Ti:Sapphire, that is able to achieve a high peak power by generating ultra-short pulses. These pulses in the femtosecond regime, when focused down to a small spot using a high-NA objective, can thus provide the high irradiance needed to generate the two-photon fluorescence signal, while at the same time only moderately bleach or even damage the tissue, since the average power the tissue is exposed to is low.

To enable TPEFM in hollow organs, one would need to deliver the excitation light through an endoscope to the site of interest, requiring a transmission of the ultra-short pulses through optical fibres. However when short pulses propagate through an optical fibre, the pulse width can change drastically due to both linear and non-linear effects, disabling two-photon excitation at the distal end of the endoscope, since the high irradiance can no longer be achieved. This unwanted pulse broadening thus has to be counteracted by appropriate means.

Different schemes have been proposed to mitigate the adversarial effects of the fibres on the short pulses. Most commonly, the femtosecond pulses are dispersed anomalously in a pre-compensation unit, either consisting of

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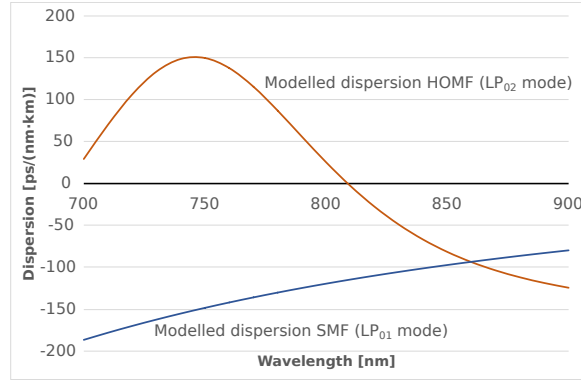


Figure 1. Dispersion properties of SMF and HOMF.

gratings,<sup>4</sup> prisms,<sup>5</sup> or a combination of both,<sup>6</sup> the latter allowing compensation of both linear and non-linear dispersion. However, all these solutions crucially depend on a well-aligned free-space optical setup, which hinders their adoption in out-of-the-lab applications.

A second approach to mitigate dispersion is to use fibres with no or negligible dispersion at the wavelength of interest, such as large mode area photonic band gap fibres (with no non-linear dispersion, but still requiring pre-compensation of linear dispersion),<sup>7</sup> or hollow core fibres (with neither linear nor non-linear dispersion).<sup>8</sup> A slightly different approach, but still using photonic crystal fibres, is the actual use of the non-linearities in the fibre to enable even shorter pulses, albeit still requiring linear dispersion compensation units.<sup>9</sup>

A novel idea for dispersion management, in the family of the pre-compensation schemes outlined above, is to use a higher order mode fibre with anomalous dispersion for the  $LP_{02}$  mode to compensate for the normal dispersion commonly exhibited by single mode fibres guiding the light in the fundamental  $LP_{01}$  mode.<sup>1,10</sup> This has the advantage that the dispersion management can be done all-fibre, not requiring any alignment sensitive free-space optics, thus being a promising candidate to be used in out-of-the-lab settings.

In this paper, we follow this approach and show the feasibility of this configuration for efficient TPEFM by recording high resolution images of fluorescent beads as a proof of concept.

## 2. METHODS

### 2.1 Dispersion Management

As it has been previously reported,<sup>1,10</sup> the  $LP_{02}$  mode from a Higher Order Mode Fibre (HOMF) can provide adequate anomalous (negative) waveguide dispersion to compensate the normal (positive) material dispersion from a Single Mode Fibre (SMF). The fibre assembly we thus use to deliver the pulse to the microscope setup consists of 81.5 cm of SMF (ClearLite 780-11, OFS Fitel, Denmark), spliced to a 94.5 cm HOM (ditto), at the onset of which a Long-Period-Grating (LPG) is engraved, used as a mode converter to couple the  $LP_{01}$  into the  $LP_{02}$  mode in the HOMF. This coupling is only efficient at a wavelength range of  $770 \pm 10$  nm. We calculated the required length of the two fibres as follows:

The dispersive properties of both fibers have been provided by *OFS Fitel, Denmark* and are shown in figure 1. At 770 nm center wavelength the SMF exhibits normal dispersion with  $D = -135.844 \text{ ps}/(\text{nm} \cdot \text{km})$ ,  $\beta_2 = +42.75 \text{ ps}^2/\text{km}$  and  $\beta_3 = +0.023 \text{ ps}^3/\text{km}$ . Here,  $\beta_2$  is the Group Velocity Dispersion (GVD) or second order dispersion and  $\beta_3$  is the Third Order Dispersion (TOD). GVD is related with parameter  $D$  from:  $D = -\frac{2\pi c}{\lambda^2} \beta_2$  and TOD refers to higher order dispersion and is related to the dispersion slope  $S = (\frac{2\pi c}{\lambda^2})^2 \beta_3$ . In addition, the dispersive properties for the  $LP_{02}$  mode of the HOMF is given as:  $D = +115.57 \text{ ps}/(\text{nm} \cdot \text{km})$ ,  $\beta_2 = -36.38 \text{ ps}^2/\text{km}$ ,  $\beta_3 = -0.22 \text{ ps}^3/\text{km}$ .

The Group Delay Dispersion (GDD)\* for these two fiber lengths was matched to be  $+34848 \text{ fs}^2$  for the SMF and  $-34378 \text{ fs}^2$  for the HOMF, respectively. Since we deal with ultra-short pulses, the influence of TOD on the

\*Group Delay Dispersion (GDD) refers to GVD multiplied by the length of the fibre.

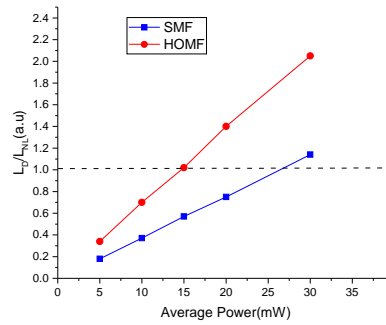


Figure 2. Ratio of dispersion length ( $L_D$ ) and non-linear length ( $L_{NL}$ ) at several input average powers for both SMF and HOMF. The dash line represents the limit for linear and non-linear pulse propagation.

pulse broadening is also significant. However, TOD can be further reduced by decreasing the total length of the fiber module. Therefore, we only used 1.76 m of total fibre length.

We briefly revisit the concepts of Dispersion length,  $L_D$ , and non-linear length,  $L_{NL}$ , in order to verify linear dispersion is indeed dominant in our setup.<sup>1</sup>

$$L_D = \frac{2\pi c T_0^2}{\lambda^2 |D|} \quad (1)$$

$$L_{NL} = \frac{\lambda A_{eff}}{2\pi n_2 P_0} \quad (2)$$

Here,  $\lambda$  is the wavelength of light,  $D$  is the dispersion parameter,  $T_0$  is the pulse width measure at FWHM,  $A_{eff}$  is the effective area,  $P_0$  is the peak power and  $n_2$  is the non-linear coefficient. A pulse has broadened to  $\sqrt{2}$  of its initial pulse width after travelling a distance  $L_D$  in the waveguide. Likewise, the  $L_{NL}$  is related to the maximum phase shift a pulse can experience when travelling a certain distance in a waveguide.

Propagation can be considered to be linear when  $L_D$  is smaller than  $L_{NL}$ . Figure 2 shows the ratio  $L_D/L_{NL}$  for both the SMF and HOMF in our fibre assembly, and it can be seen that only for an average power lower than 15 mW, indeed the linear effects are dominant. We stayed under this power limits for all our experiments. Group Velocity Dispersion (GVD) is thus the dominant effect and broadens the pulse by changing its temporal width.<sup>11</sup> Non-linear Self-Phase Modulation (SPM) still adds an extra phase shift on the pulse, changing its spectral width and, as a consequence, broadens the pulse,<sup>12</sup> albeit only slightly in our experiment, as we will show.

## 2.2 Imaging Setup

Fig. 3 shows the experimental set-up of the two-photon microscope. Femtosecond pulses of 18 fs duration are generated using a Ti:Sapphire laser at 800 nm centre wavelength, 75 MHz repetition rate (femtosecond compact, femtolasers, Austria). Since the LPG module can only efficiently couple light from the  $LP_{01}$  to the  $LP_{02}$  mode at  $770 \pm 10$  nm,<sup>1</sup> we filtered the laser output with a bandpass filter (FB770-10, Thorlabs, USA) to obtain the appropriate spectrum (measured with an S2000, Ocean Optics, USA). We measured the pulse duration after the filter using an autocorrelator (femtometer, femtolasers, Austria) to be 40 fs. The output collimated laser beam was coupled into the fibre assembly described above. The output beam from the fibre module was collimated with an objective lens (NA 0.25) and coupled into the galvanometric scanner assembly (GVS012, Thorlabs, USA) before the microscope. Two achromatic relay lenses (AC) are used to expand the beam, that then propagates through a long pass filter (FEL0700, Thorlabs, USA) and is reflected at the dichroic mirror (HC720SP, AHF, Germany) and fills the back aperture of a water dipping microscope objective with an NA of 1.0 and  $20\times$  magnification (XLUMPLFLN, Olympus, Japan). The fluorescence is collected using the same objective, passes the dichroic mirror and a short pass emission filter (F75-720, AHF, Germany) and is detected using a PMT (H5784-01, Hamamatsu, Japan). The scanning and collection system was controlled using a I/O box (USB-6353, National Instruments, USA) and a custom built LabVIEW software.

To measure the power dependency on the irradiance, a sample of Rhodamine B dye has been examined. We then turned to image  $6 \mu\text{m}$  fluorescence beads mounted on a glass slide (F-14807 from set F-24633, ThermoFisher)

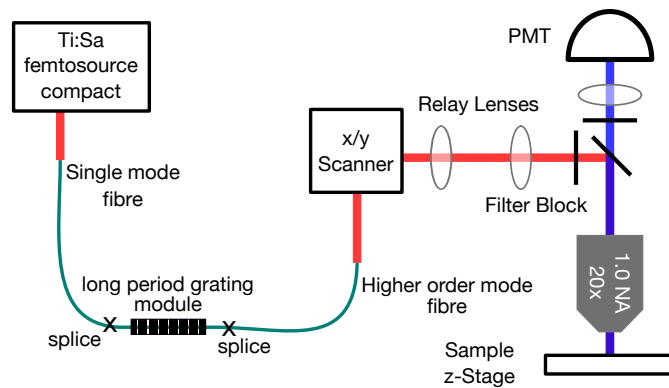


Figure 3. Experimental Set-up of a fibre-based microscope

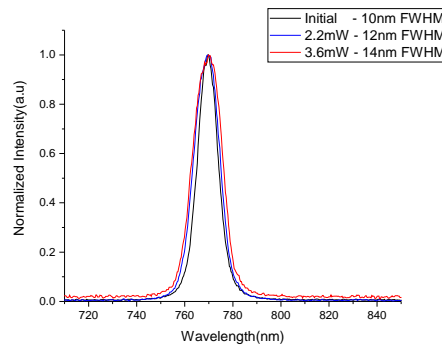


Figure 4. Spectrum of the laser before and after the fibre, at different input powers.

that consist of two parts: a fluorescence ring and a staining throughout. The fluorescence ring is dark red with a single-photon excitation wavelength at 660 nm and an emission peak at 680 nm. The stain throughout is green with a single-photon excitation at 505 nm and an emission peak at 515 nm.

### 3. RESULTS

The total power loss in the fibre assembly was measured to be 2.2 dB.

The spectrum of the pulse was measured before and after the fibre module for an average input power of 3.6 mW and 2.2 mW (taking into consideration the 2.2 dB splice loss and 7.1% losses from the input coupling lens objectives). A comparison between the three spectra is demonstrated in figure 4. The spectral broadening is about  $5 \pm 1$  nm.

To verify we indeed observe two-photon fluorescence, we measured the fluorescence emission for Rhodamine B as a function of average power, as shown in figure 5,. The power was increased from 0.4 mW to 1.2 mW in steps of 0.2 mW giving a slope value of 2.11.

The sample with the fluorescence beads was measured at 1.3 mW average power, measured before the 'X,Y' scanner, as shown in 6.

### 4. DISCUSSION

The LPG provides efficient mode conversion from  $LP_{01}$  to  $LP_{02}$  only at  $770 \pm 10$  nm. Since the Ti:Sapphire laser did not provide any functionality to tune it in a wavelength different than 800nm, the bandpass filter utility was to eliminate all the unwanted out of band radiation and at the same time to allow high transmission in the region of 770nm. A center wavelength different than 770nm would reduce the coupling behaviour between

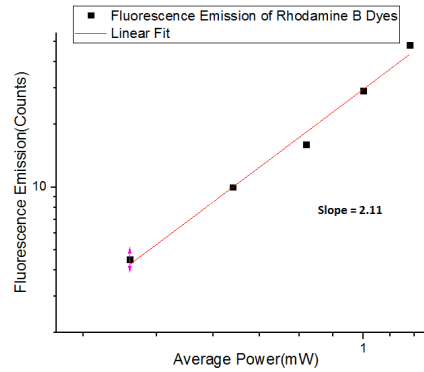


Figure 5. Fluorescence emission dependence on the input power.

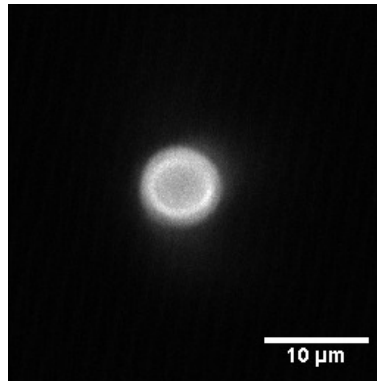


Figure 6. Image of a fluorescent bead.

these modes and therefore lead to an inefficient mode conversion which in turns leads to an inefficient dispersion compensation. A laser tuned to 770 nm would be better suited for our application. In general, however, a freely tuneable excitation laser would be desirable, since such a laser could be tuned to the specific excitation wavelength of the targeted fluorophore. This would pose a problem for our dispersion management solution, since the LPG we used is not tuneable in wavelength. Further options to couple into the  $LP_{02}$  mode of a HOMF would thus need to be conceived.

Linear propagation was determined by taking into consideration equation 1 and 2 where  $L_D$  was found to be smaller than  $L_{NL}$  pointing out the fact that linear dispersion is the most dominant effect. Figure 2 shows the Ratio of dispersion and non-linear length as a function of average power for 40 fs pulse. It can be clearly seen by the graph that for the case of SMF, GVD is the most dominant effect at average power lower than 25 mW. For the HOMF the linear limit is reduced to 15 mW, underpinning the fact that non-linear effects and specifically SPM are more of concern in the HOMF than the SMF. This is because the HOMF exhibits smaller values of  $A_{eff}$  for the  $LP_{02}$  mode than the SMF for the  $LP_{01}$  mode. We therefore kept the average powers low. However, even for low input power levels, the influence of SPM broadens the pulse spectrum by  $5 \pm 1$  nm, as can be seen in figure 4. Theoretical calculations show that after propagation in 0.815 m of SMF, the pulse duration increases from 40 fs to 2.7 ps taking into account only second order dispersion. Since both fibres exhibits the same amount of GDDs but with opposite signs, an efficient GVD compensation is expected at the output of the HOMF. The impact of the TOD remains, however, this can be further reduced by decreasing the total fiber length.

Despite all of these obstacles, we could easily record two-photon fluorescence images. In figure 5 the non-linear power dependence of fluorescence emission for Rhodamine B dyes revealed a slope of 2.11. The small aberration from 2.0 is probably due to the error that might exist from the background signal. The two photon fluorescence image of a fluorescent bead in figure 6 depicts the bead with high resolution and shows high fluorescence intensity with only 1.3 mW average excitation power at the sample.

We are focussing the  $LP_{02}$  mode directly onto the sample, without converting it back to the fundamental mode. Smith et al.<sup>13</sup> claimed that the  $LP_{02}$  mode is providing better lateral resolution than the  $LP_{01}$  in a non-linear process. However, Lee et al.<sup>14</sup> showed in their calculations that the  $LP_{02}$ , when focused, generates a ring focus with a void in the center. In the latter case, lateral resolution would indeed be worse than for the  $LP_{01}$ , however, as we could show, the resolution is still on the order of one micrometer. Such a Bessel-like beam or a ring focus might even have potential for imaging applications such as lightsheet or STED microscopy.

## 5. CONCLUSION

We demonstrate a technique of delivering ultrashort pulses through a fibre for TPEFM. Ultrashort pulses have been delivered at the output of 1.76 m all-fibre-based configuration which consists of a SMF-LPG-HOMF. Images of fluorescence beads with diameter of 6  $\mu\text{m}$  were taken and analysed, pointing out that the scheme is capable of providing high resolution which is an important asset for detecting tumour tissues.

In terms of complexity and applicability, the all-fibre-based scheme is more favourable than pre-compensation schemes where more optical components and free-space alignment are needed.

Last but not least, the all-fibre-based configuration presented in this work is a very powerful tool and could potentially be used for endoscopic cancer diagnosis. It needs only few seconds to retrieve an image and to reveal TPEFM biomarkers, giving doctors the opportunity to facilitate their treatment in an early diagnosis process.

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